Title Page

Upregulated sirtuin 1 by miRNA-34a is required for smooth muscle cell differentiation from pluripotent stem cells

Running Title: miR-34a and SirT1 in smooth muscle differentiation

Xiaotian Yu[1,2#], Li Zhang[3#], Guanmei Wen[1], Hanqing Zhao[1], Le Anh Luong[1], Qishan Chen[1,3],Yuan Huang[1,3], Jianhua Zhu[3], Shu Ye[1], Qingbo Xu[4], Wen Wang[2*] and Qingzhong Xiao[1*]

[1]Centre for Clinical Pharmacology, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, UK;
[2]Institute of Bioengineering, Queen Mary University of London, London E1 4NS, UK;
[3]Department of Cardiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, 310003, Zhejiang, China;
[4]Cardiovascular Division, King's College London British Heart Foundation Centre, London SE5 9NU, UK

[#]X Yu and L Zhang contributed equally to this study

*Correspondence to:

Professor Wen Wang, Institute of Bioengineering, Queen Mary, University of London, London E1 4NS, United Kingdom. Tel: +44 (0)20 7882 8871; E-mail: wen.wang@qmul.ac.uk

Dr Qingzhong Xiao, Centre for Clinical Pharmacology, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Heart Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom. Tel: +44(0)2078826584; E-mail: q.xiao@qmul.ac.uk

SUMMARY

microRNA-34a (miR-34a) and sirtuin 1 (SirT1) have been extensively studied in tumor biology and longevity/aging, but little is known about their functional roles in smooth muscle cell (SMC) differentiation from pluripotent stem cells. Using well-established SMC differentiation models, we have demonstrated that miR-34a plays an important role in SMC differentiation from murine and human embryonic stem cells. Surprisingly, SirT1, one of the top predicted targets, was positively regulated by miR-34a during SMC differentiation. Mechanistically, we demonstrated that miR-34a promoted differentiating stem cells arrest at G0/G1 phase, and observed a significantly decreased incorporation of miR-34a and SirT1 RNA into Ago2-RISC complex upon SMC differentiation. Importantly, we have identified SirT1 as a transcriptional activator in the regulation of SMC gene programme. Finally, our data showed that SirT1 modulates the enrichment of H3K9 tri-methylation around the SMC gene promoter regions. Taken together, our data reveal a specific regulatory pathway that miR-34a positively regulates its target gene SirT1 in a cellular context-dependent and sequence specific manner, and suggest a functional role for this pathway in SMC differentiation from stem cells.

Key words: microRNAs, miR-34a, sirtuin 1, stem cells, smooth muscle cells, cell differentiation.

Introduction

Regenerative medicine is an emerging area of multidisciplinary research in cell engineering and molecular biology. Its ultimate goal is to repair, replace or regenerate cells, tissues or organs that are lost or damaged due to disease, injury or ageing. Recent advances in this field have been greatly influenced by improved understanding of tissue engineering, stem cell biology, tissue turnover and replacement in adult mammals and in embryonic development. Pluripotent stem cells, including embryonic stem (ES) cells, have been studied extensively in regenerative medicine because of their distinctive capacity to self-renew and to differentiate into a wide range of specialized cell types, including vascular endothelial cells[1, 2] and smooth muscle cells (SMCs) [3-6]. SMC differentiation is a critical process during cardiovascular system formation and development, and plays a significant role in cardiovascular diseases, such as atherosclerosis. The understanding of the transcriptional regulatory circuitry of SMC differentiation is, therefore, fundamental in cardiovascular system development and to realize the therapeutic potential of stem cells. However, the detailed molecular mechanisms of SMC differentiation from stem cells have not been fully clarified.

A novel class of gene expression regulators, microRNAs (miRNAs), have been recently identified in almost all the species and implicated in various aspects of embryonic development, cellular homeostasis and disease. Traditionally, mature miRNAs are thought to suppress gene expression by inducing mRNA cleavage[7] or mRNA decay[8] or by inhibiting mRNA translation[9]. Strikingly, evidence has also been shown that miRNAs can upregulate target gene expression under specific circumstance[10, 11]. Interestingly, miRNAs have been suggested to play an important role in cell/tissue specification due to the fact that miRNAs are recognized as a distinct class of biological regulators with conserved functions and that many miRNAs are expressed in a tissue-specific manner[12]. By disrupting dicer[13] or drosha[14] (both are the rate-limiting enzymes involved in the miRNA biogenesis) expression in ES cells, miRNAs are seen to play a role in stem cell self-renewal and differentiation. The significance of miRNAs in cardiovascular development has been revealed in a study of Dicer-deficient mice which showed that the loss of miRNAs caused severe impairment of heart and blood vessel development[15]. Furthermore, it has been shown that deletion of Dicer in vascular smooth muscle caused late embryonic lethality at

embryonic day 16 to 17 due to thinner vessel walls, impaired contractility and hemorrhage because of the decreased SMC proliferation and differentiation[16]. However, the significance and exact role of individual miRNAs in SMC differentiation remains to be elucidated. In the present study, we demonstrate for the first time that miR-34a plays an important role in human and murine ES cell differentiation towards the SMC lineage *in vitro* and *in vivo*. Unexpectedly, our data also reveal that miR-34a positively regulates its target gene, deacetylase sirtuin 1 (SirT1), which is identified as a potential transcriptional activator of SMC genes.

Results

miR-34a is significantly upregulated during SMC differentiation from mouse ES cells

Murine ES cells were replated into collagen-coated flasks and cultured in the absence of leukaemia inhibitory factor (LIF) for 2 to 8 days to allow SMC differentiation as described previously[3-6]. As expected, SMC specific markers, such as SMαA and SM-MHC, were expression upon cell differentiation (**Figure S1**). To identify potential miRNA candidates for SMC differentiation, total RNA including small RNA were harvested and subjected to miRNA microarrays analysis (Miltenyi Biotec GmbH). Data from the microarrays analysis revealed that, except miR-294, all other five members of miR-290 family, which is reported to be mouse ES cell specific miRNA cluster[17], were dramatically downregulated at day 8, further confirming cell differentiation (**Table S1**). SMC differentiation related miRNAs, such as miR-143 and 145, and cardiac/muscle miR-133, were increased in our SMC differentiation model, while miR-21 involved in SMC proliferation was undetectable at early stage of SMC differentiation (day 4) but dramatically increased at late stage of SMC differentiation (day 8), indicating that some miRNAs (eg. miR-143/145) may initiate SMC differentiation whereas others (eg. miR-21) may play important roles in the late stage of SMC differentiation where differentiated SMC proliferation is required to complete the SMC differentiation process. No significant changes were observed for other cell lineages specific miRNAs, such as miR-146 for T lymphocyte, miR-107 for monocytes, miR-203 for epidermal cell, miR-206 for myogenic cells, miR-124 for neural cells and miR-126 for endothelial cells (**Table S1**). Taken together, these findings confirm the specificity of SMC differentiation. Interestingly, the microarray analysis showed miR-34a, a reported cell cycle mediator[18], was upregulated to a greater magnitude than the best known SMC differentiation related miRNAs miR-143 and 145, during SMC differentiation, which was further confirmed by real-time RT-PCR analysis (**Figure 1A**), indicating that miR-34a may play an important role in our SMC differentiation system.

Important role of miR-34a in SMC differentiation from ES cells in vitro

To investigate whether miR-34a induces SMC differentiation, gain-of-function experiments were conducted by using Pre-miR™ mmu-miR-34a miRNA Precursor

(Ambion) in differentiating ES cells. The experiments showed that miR-34a overexpression induced the expression of SMC-specific genes (*SMαA, SM22α, h1-calponin* and *SM-myh11*) (**Figure 1B**). Western blot analysis revealed that there was also increased protein production of these genes (**Figure 1C**). To further test if miR-34a expression was essential for SMC differentiation, lose-of-function experiments by using Anti-miR™ miR-34a inhibitoror (Ambion) were performed in differentiating ES cells. The study showed that knockdown of miR-34a inhibited expression of smooth muscle differentiation specific markers at both RNA (**Figure 1D**) and protein levels (**Figure 1E**). These data suggest an important role of miR-34a in SMC differentiation from stem cells.

To further understand the molecular mechanism by which miR-34a regulates SMC differentiation, microarray analyses (Beadchip: mouseWG-6_V2_0_R3_11278593-A) was conducted by GENERGY Bio to examine the gene expression profiling in differentiating SMCs affected by miR-34a over-expression. Expectedly, data summarised in **Table S2** showed that a panel of SMC specific genes such as CNN1, Acta2, Tagln2, Aebp1, Smtn, Des, Cald1, Mylk, Myh11, and Vcl (yellow-highlighted) were up-regulated by miR-34a over-expression. Importantly, Several SMC specific transcription factors including SRF, Myocd and MEF2c that were reported to be activated in our SMC differentiation system[3, 19] were also significantly up-regulated by miR-34a, alongside SirT1 (Table S2). Therefore, we decided to investigate if these factors worked in concert. Over-expression of miR-34a up-regulated SRF, myocardin and MEF2c, whereas knockdown of miR-34a down-regulated these genes (**Figure S2**), suggesting that miR-34a works in concert with these SMC transcription factors during SMC differentiation from stem cells.

miR-34a is involved in SMC differentiation in vivo

To facilitate our *in vivo* cell implantation study, miR-34a over-expressing (pLL3.7-GFP-miR-34a) and control (pLL3.7-GFP) ES cell lines were generated by using miR-34a over-expression (pLL3.7-GFP-miR-34a) and control (pLL3.7-GFP) lentivirus, respectively. GFP-positive cells with high purity (**Figure S3A**) were sorted out and maintained in ES cell culture medium. No significant differences were observed between the sorted cells and their parent ES cells in terms of morphology, self-renew and pluripotency when they were cultured in ES cell culture medium for up to five passages (**data not shown**). As expected, compared to control ES cells, the

expression levels of miR-34a in miR-34a overexpressing ES cells were slightly increased at day 0 (undifferentiated cells), but significantly up-regulated at day 8 of differentiation (**Figure S3B**), suggesting that miR-34a expression levels were successful up-regulated in these cells during SMC differentiation. Consequently, more SMCs were differentiated from miR-34a over-expressing ES cells (**Figure S3C**), further confirming a promotive role of miR-34a in SMC differentiation. To further explore the functional importance of miR-34a in SMC differentiation *in vivo*, pLL3.7-GFP and pLL3.7-GFP-miR-34a ES cells were subcutaneously injected into C57BL/6J mice with 100ng/ml of PDGF-BB to promote *in vivo* SMC differentiation as described in our previous study[3, 4]. Immunofluorescence staining with antibody against SM-MHC showed that more SM-MHC-positive cells were presented in the implants of miR-34a overexpressing ES cells (**Figure 2A and 2B**). As expected, the majority of cells in the Matrigel implants were GFP-positive (green signal), indicating its exogenous origins (**Figure 2A**). Importantly, the expression levels of miR-34a, SMαA, SM-MHC and SirT1 in the Matrigel implants of pLL3.7-GFP-miR-34a ES cells were significantly higher than that of control pLL3.7-GFP ES cells as demonstrated by immunofluorescence staining (**Figure 2A**), RT-qPCR (**Figure 2C**) and western blot (**Figure 2D**), respectively, further confirming the efficiency of miR-34a over-expression and the importance of miR-34a in SMC differentiation. Taken together, these data clearly suggest an important role of miR-34a in SMC differentiation from stem cells *in vitro* and *in vivo*.

miR-34a also plays an important role in SMC differentiation from human ES cells

To translate our finding that miR-34a plays a major regulated role in SMC differentiation observed from murine cell system to human, human ES cells were induced to differentiate towards SMCs by using a similar differentiation protocol as used in the mouse ES cell differentiation study. Data showed that we have successfully differentiated human ES cell into SMCs as demonstrated by real-time RT-PCR (**Figure S4A**), Western blot (**Figure S4B**), immunofluorescence staining (**Figure S4C**) and flow cytometry (**Figure S4D**) analyses. Along with SMC marker gene activation, miR-34a was significantly increased during SMC differentiation from human ES cells (**Figure S5A**), implying that miR-34a may also play a role in human SMC differentiation. Importantly, miR-34a over-expression (**Figure S5B and S5C**) significantly up-regulated, while miR-34a inhibition (**Figure S5D and S5E**)

dramatically down-regulated SMC marker gene expressions in differentiating human ES cells, further demonstrating a critical role of miR-34a in murine as well as human SMC differentiation.

Target gene, SirT1, is positively regulated by miR-34a through the binding sites within 3'UTR

To further investigate the mechanisms by which miR-34a regulate SMC differentiation, the potential mRNA targets of miR-34a were scrutinized. SirT1 was emerged as one of the top targets of miR-34a in several computational algorithmic databases, and two highly conserved binding sites for miR-34a have been identified within SirT1 3'UTR (**Figure 3A**). Unexpectedly, SirT1 gene expressions were significantly up-regulated (**Figure 3B**), and displayed a positive correlation with the gene expression levels of miR-34a during SMC differentiation (**Figure 1A**), suggesting that miR-34a may positively regulate this target gene, SirT1. Such notion has been firmly supported by the findings that SirT1 gene and protein levels were significantly up-regulated or down-regulated by over-expression or inhibition of miR-34a in the differentiating ES cells (**Figure 3C and 3D**), respectively. These data indicate that SirT1 is positively regulated by miR-34a directly or indirectly. To confirm such possibility, the 3'UTR of SirT1 which contained the two binding sites of miR-34a was cloned into a luciferase reporter. Data from our miRNA reporter assay showed that the activity of luciferase from construct harbouring the SirT1 3'UTR was significantly up-regulated by miR-34a over-expression (**Figure 3E**). Most importantly, we have observed that the binding site 2 is responsible for SirT1 3'UTR reporter activity up-regulation mediated by miR-34a by using site-directed mutagenesis of the predicted individual binding site or all two binding sites for miR-34a in the SirT1 3'UTR reporter (**Figure 3E**). Altogether, above data has firmly confirmed that SirT1 is a true mRNA target of miR-34a, which is positively regulated by miR-34a during SMC differentiation.

miR-34a differently regulates SirT1 gene expression in ES cells and terminally differentiated SMCs

To investigate if the observed phenomena that miR-34a directly up-regulated its target gene, SirT1, expression during SMC differentiation is cellular context specific, we conducted miR-34a over-expression or inhibition experiments and luciferase activity assays in naïve stem cells (undifferentiated ES cells) and terminally differentiated

SMCs (we previously reported that day 8 differentiated SMCs are fully/terminally differentiated SMCs[19]), respectively. Data showed that SirT1 3'UTR reporter activity was significantly down-regulated by miR-34a over-expression, but up-regulated by miR-34a inhibition at naïve stem cell stage (**Figure 4A**). As expected, SirT1 gene expression was significantly increased by over-expression of miR-34a, while decreased by miR-34a knockdown (**Figure 4B**), suggesting that miR-34a regulates SirT1 gene expression is cellular context dependent. In the previous section, we have successful demonstrated the miR-34a bindings sites are required for miR-34a mediated SirT1 gene regulation (**Figure 3E**). We further wondered if the miR-34a binding site is sufficient for miR-34a mediated SirT1 gene expression. To this aim, we constructed a luciferase reporter with an artificial miR-34a binding site on its 3'UTR, without surrounding RNA sequence and structure of SirT1 3`UTR, designated as pmiR-Luc-miR-34a binding site, and performed luciferase activity assay. We found that miR-34a slightly up-regulated this reporter activity, but could not reach significance (**Figure 4C**), indicating that the surrounding RNA sequence and/or structure of SirT1 3'UTR is also required for miR-34a mediated SirT1 gene up-regulation.

We have previously reported miR-200c mediates endothelial cell differentiation by targeting transcription factor ZEB1[20] which contains no miR-34a binding site within 3'UTR. Expectedly, we observed no effects of miR-34a over-expression on ZEB1 gene reporter activity (a non-miR-34a target) (**Figure S6A**), confirming that miR-34a specifically up-regulates SirT1 gene expression during SMC differentiation. To further distinguish if our observation that up-regulation of SirT1 by miR-34a during SMC differentiation is a universal phenomenon or a specific event, two additional experiments were conducted. We first performed luciferase assays with the non-miR-34a reporter (pmiR-Luc-ZEB1) in conjunction with miR-200c over-expression in naïve stem cells and terminally differentiated SMCs, respectively. We found that miR-200c negatively regulated ZEB1 gene reporter activity in both cells (**Figure S6B and S6C**), which is consistent with our previous observation in endothelial cell differentiation. Moreover, it is well-known that 12-O-tetra-decanoylphorbol-13-acetate (TPA) can induce monocyte differentiation through regulating cell cycle progression. We then applied this model to investigate if up-regulation of SirT1 by miR-34a may (or may not) be specific to differentiating stem cells. While miR-34a expression levels were clearly up-regulated by TPA during monocytes differentiation,

no significant changes in terms of SirT1 expression levels were observed during the same process (**Figure S6D**), implying no significant involvement of miR-34a in regulation of SirT1 gene expression in this monocyte differentiation model. Taken together, above data clearly suggests that up-regulation of SirT1 by miR-34a during SMC differentiation from ES cells is a specific event which is likely dependent on specific cellular context, miRNA binding sites and surrounding sequence, and/or other RNA binding proteins.

Less miR-34a and SirT1 mRNA were incorporated into Ago 2-RISC complex upon differentiation

To further investigate the molecular mechanism by which miR-34a up-regulates SirT1 gene expression upon SMC differentiation, we conducted the RNA-IP assays with Ago 2 antibody in both undifferentiated stem cells and differentiated SMCs. Expectedly, no significant difference in terms of Ago 2 protein levels between undifferentiated ES cells and differentiated SMCs was observed (Input samples, **Figure 4D**), and the immunoprecipitation efficiencies in both cells are comparable (Ago2-IP and IgG-IP samples, **Figure 4D**). Moreover, compared to IgG control, Ago2 antibody could specifically pull-down miR-34a as well as SirT1 RNA with mean enrichment folds of 26 v.s 2.1 (ESCs v.s dSMCs) for miR-34a, 16.4 v.s 3.94 for SirT1 3'UTR around binding site 1, and 26.5 v.s 2.1 for binding site 2, respectively (**Figure 4F**). Importantly, the enrichment levels of miR-34a and SirT1 3'UTR RNA (spanning around miR-34a binding sites 1 and 2) in undifferentiated stem cells (ESCs) were much higher than that of differentiated SMCs (dSMCs) (**Figure 4E**), indicating that much less miR-34a and SirT1 3'UTR were loaded into Ago 2 RISC complex upon SMC differentiation.

miR-34a promotes cell cycle arrest at G0/1 in differentiating ES cells

Several elegant studies conducted by Joan A. Steitz and colleagues have famously revealed that the miRNA-target gene regulatory machinery will switch from translation repression to activation when the cells have been arrested at G0/1 phase[10] and miRNAs can regulate target gene translation activation in quiescent cells[11]. We therefore wondered if the differentiating cells underwent such cell cycle arrest. As expected, cell cycle analyses revealed that differentiating cells were gradually arrested at G0/1 phase during SMC differentiation (**Figure S7A**). Importantly, such arrest has been significantly reinforced by miR-34a over-expression (**Figure S7B**). Moreover,

data from apoptosis analysis and BrdU incorporation assays showed that miR-34a plays no significant role in cell apoptosis and death (**Figure S7C**), while much less cells were entering S-phase upon miR-34a over-expression (**Figure S7D**). Taken together, these data suggests that the promotive effects of miR-34a on cell cycle arrest at G0/1 is, at least partially, responsible for the SirT1 gene up-regulation by miR-34a during SMC differentiation. To further confirm if miR-34a up-regulates SirT1 gene expression is specific to G0/G1 cell cycle stage, differentiating SMCs were arrested at G0/G1 phase by cell cycle arrest inducer aphidicolin[10] and luciferase activity assays were conducted. As expected, incubation of differentiating SMCs with 2μg/ml aphidicolin for 24 to 28 hours significantly increased cells arrest at G0/G1 stage, which was further increased in the presence of miR-34a over-expression (**Figure S7E**). Consequently, SirT1 3'UTR reporter activity was significantly up-regulated by aphidicolin and miR-34a over-expression in a similar manner (**Figure S7F**).

SirT1 activation is required for miR-34a mediated SMC gene expressions

We have demonstrated nicely that SirT1 is the mRNA target of miR-34a during SMC differentiation. To investigate the potential role of SirT1 in SMC differentiation, SirT1 over-expression in the differentiating ES cells was conducted by using SirT1 over-expression plasmid (pcDNA3.1-SirT1[21], a kind gift from Dr. Hang Shi, Wake Forest University School of Medicine, North Carolina, USA). Data showed that over-expression of SirT1 significantly up-regulated SMC specific marker expressions (**Figure 5A and 5B**), suggesting that SirT1 over-expression can recapitulate the effects of miR-34a during SMC differentiation from ES cells. We further hypothesised that SirT1 activity is required for miR-34a mediated SMC differentiation. To test this, control (pLL3.7-GFP) or miR-34a over-expressing (pLL3.7-GFP-miR-34a) ES cells were induced to differentiate into SMCs for 4 days in the absence or presence of SirT1 specific inhibitor. Data shown in **Figure 5C** revealed that while SirT1 inhibition (2^{nd} columns) or miR-34a over-expression (3^{rd} columns) alone in the differentiating ES cells were significantly down-regulated or up-regulated various SMC specific gene expression, respectively, inhibition of SirT1 almost completely abolished SMC specific gene up-regulations induced by miR-34a over-expression (4^{th} columns). Such observation was further confirmed in SirT1 knockdown experiments by using SirT1 specific siRNA (**Figure 5D**), suggesting that miR-34a regulates SMC gene expression during SMC differentiation from ES cells through activation of SirT1. It has been reported that SirT-1 agonist resveratrol

promotes osteogenic differentiation of mesenchymal stem cells[22, 23]. Consistently, our data revealed that resveratrol also significantly increased SMC specific gene expression with the best concentration of 5μM (**Figure 5E**), further supporting a role of SirT1 activation in SMC differentiation.

SRF binding site is required for SirT1 mediated SMC gene expression

SirT1 has been shown to up-regulate SMC specific gene expression. To investigate if SirT1 over-expression will activate the specific SMC gene transcription, luciferase activity assays were conducted in differentiating ES cells. The reporter gene plasmids used in the luciferase assay, pGL3-Luc-SMαA and pGL3-Luc-SM22α, were previously designed and cloned in our previous study[24]. Data shown in **Figure 6A** revealed that the overexpression of SirT1 in differentiating ES cells significantly increased SMαA and SM22α gene promoter activities, indicating that SirT1 over-expression can activate specific SMC gene promoters. It has been well-known that SRF binding element (CArG) within promoter region of SMC genes is required for transcriptional activation of SMC genes[25], we thus wondered if this is also a case for SirT1-regulated SMC gene transcriptional activation. To this aim, another set of luciferase assays using SRF binding site mutants (pGL3-Luc-SMαA-SRF^{mu} and pGL3-Luc-SM22α-SRF^{mu}) generated in our previous study[3] were carried out in differentiating ES cells. Data showed that SRF binding element mutation in pGL3-Luc-SMαA and pGL3-Luc-SM22α resulted in complete lost of their transcriptional activity in response to SirT1 over-expression (**Figure 6A**), suggesting that SirT1 regulates SMC differentiation gene expression through SRF binding site within the promoters. Moreover, ChIP assays were conducted using SirT1 antibody in the differentiating ES cells to further verify if SirT1 activates specific SMC gene transcription through direct binding to their promoters. Data shown in **Figure 6B** revealed that SirT1 directly bound to the region spanning around SRF binding element (CArG) of SMαA and SM22α gene promoters, and such binding was dramatically enhanced by SirT1 over-expression. Finally, CHIP assay data also showed that the binding capacity of SRF to SMαA and SM22α gene promoters was significantly enhanced by SirT1 over-expression (**Figure 6C**). Taken together, above findings demonstrate for the first time that SirT1 regulates SMC specific gene expressions during SMC differentiation through direct binding to the promoter region of SMαA and SM22α genes and increasing the binding ability of SRF to SMC specific gene promoters.

SMC transcription factors are transcriptionally regulated by SirT1

SRF, MEF2c and myocardin are well-known transcription factors for regulating SMC gene expression and play important roles in cardiovascular system development. Data shown in **Figure 7A** revealed that gene expression levels of all three factors were significantly regulated by SirT1 over-expression, suggesting SirT1 may have a direct role in regulation of these transcription factors during SMC differentiation. To confirm such possibility, luciferase activity assays by using SRF, MEF2c and Myocd gene reporter plasmids (pGL3 -Luc-SRF, pGL3 -Luc-MEF2c and pGL3 -Luc-Myocd) generated in our previous study[3] were conducted in the differentiating ES cells. Data showed that the SirT1 over-expression significantly increased SRF, MEF2c or Myocd gene promoter activities (**Figure 7B**), indicating that SirT1 may activate transcriptional activity of these three genes. To further investigate if SirT1 can directly bind to the promoters of SRF, MEF2c and Myocd, and its potential binding region(s) of SirT1 within these three gene promoters, CHIP assays with SirT1 antibody were conducted and two pairs of primers for each gene were used to amplify target DNA. A set of specific primers (4 pairs) spanning through the respective promoter regions of SRF, MEF2c and Myocd as described in our previous study[3] were used in our preliminary study and the best primer pairs were chosen to use in the following experiments. Data shown in **Figure 7C** revealed that SirT1 directly binds to the promoter regions between -1393 and -1274 of SRF gene, -1335 and -1263 of MEF2c gene or -708 and -620 of Myocd gene, respectively, and such binding activity was significantly enhanced by SirT1 over-expression. Taken together, these findings strongly imply that SirT1 transcriptionally regulates SMC transcription factor gene expression during SMC differentiation from stem cells.

SirT1 promotes SMC gene expression through inhibiting H3K9 methylation around SMC gene promoters

DNA and/or histone methylation is an important gene transcription regulator, and has been implicated in enormous cellular functions and various diseases. It is well-known that methylation of lysines H3K9 and H3K27 is closely associated with transcriptional repression[26]. To investigate if SirT1 affects H3K9me3 expression levels during SMC differentiation, SirT1 over-expression experiments were conducted in differentiating ES cells. Data showed that H3K9me3 protein expression levels were not regulated by SirT1 over-expression (**Figure S8A**), however their enrichments on SMαA and SM22α gene promoters were significantly inhibited by

over-expression of SirT1 (**Figure S8B**), suggesting that SirT1 regulates SMC-specific gene activation, at least partially through inhibiting H3K9 tri-methylation around SMC specific gene promoters.

Discussion

SMC differentiation from pluripotent stem cells is a complicated and poorly defined process triggered by a variety of stimulation, such as growth factors, extracellular matrix, mechanical stress and micro-environmental milieu, and governed by various molecular signal pathways. Despite enormous efforts have been put into this field in the past decade, our understandings into the molecular mechanisms underlying SMC differentiation are still far from complete. In the present study, we have advanced our knowledge of the molecular mechanism mediating SMC differentiation by uncovering an important role of miR-34a in regulating SMC specific gene expression and SMC differentiation from murine ES cells *in vitro* and *in vivo*. Such importance of miR-34a has also been confirmed in human SMC differentiation from stem cells. Surprisingly, we have identified SirT1 as the mRNA target of miR-34a, and demonstrated that miR-34a positively regulates its target gene, *SirT1*, during SMC differentiation. Mechanistically, we have found that miR-34a promotes cell cycle arrest at G0/1 phase, and observed a significant decreased incorporation of miR-34a and SirT1 RNA into Ago2-RISC complex upon SMC differentiation. We have also clearly defined that SirT1 is a potential transcription activator of SMC-specific gene regulation. Moreover, our data also revealed that the SRF binding site(s) within SMC specific gene promoters is required for SirT1 mediated SMC gene transcriptional activation, and SirT1 regulates SMC transcription factors (*SRF*, *MEF2c* and *Myocd*) at transcriptional level. Finally, we have observed that SirT1 regulates SMC gene expression through modulating the histone methylation status around SMC-specific gene promoters. Taken together, data provided in the current study strongly suggests that miR-34a is a powerful SMC differentiation modulator by positive regulating its target gene, *SirT1*, a molecule functioning as a potential SMC-specific gene transcriptional activator.

miR-34a and SMC differentiation

In mammalians, the miR-34 family comprises three processed miRNAs that are encoded by two different genes: miR-34a is encoded by its own transcript, and miR-34b and miR-34c share a common primary transcript[27]. In 2007, several laboratories independently reported that members of the miR-34 family are direct p53 targets, and their upregulation induces apoptosis and cell-cycle arrest[18, 28, 29]. Strikingly, the most highly enriched gene ontology category among the upregulated transcripts induced by miR-34a overexpression was ‘‘cell cycle’’[28], which strongly indicates that miR-34a is

a major player in the regulation of cell cycle progression. Considering cell cycle arrest or exiting from cell cycle progression is a critical process during cell differentiation, miR-34a could be a major regulator in cell differentiation from stem cells. In addition, similar to other p53-target genes, miR-34a may be an important regulator for other signalling pathways involved in normal embryos development, cell differentiation, apoptosis, senescence, proliferation and various diseases including cancer and cardiovascular diseases. Importantly, it has been recently reported that suppression of somatic cell reprogramming into pluripotent cells by miR-34a was due, at least in part, to repression of pluripotency genes, including Nanog, Sox2 and N-Myc[30], which strongly suggests that miR-34a could play an important role in stem cell differentiation. Evidently, recent studies have suggested an important role of miR-34a in neural differentiation from neural stem cells[31] and megakaryocytic differentiation from bipotent K562 human leukemia cells[32]. miR-34a has also been identified as a critical cell-fate determinant in early-stage dividing colon cancer stem cells[33]. However, the functional role of miR-34a in SMC differentiation remains to be explored. By using our well-established SMC differentiation system and miRNA array technique, miR-34a was emerged as a top potential miRNA candidate to regulate SMC differentiation. By utilizing miRNA gain/lose-of function analyses, we confirmed a critical role of miR-34a in SMC differentiation from murine and human ES cells *in vitro*. Furthermore, we also provide clear evidence to support that miR-34a plays an important role in embryonic SMC differentiation *in vivo* by generating miR-34a over-expressing ES cell lines and using another well-established *in vivo* Martigel implantation model. These observations clearly implied that miR-34a is a SMC differentiation regulator.

Up-regulation of SirT1 by miR-34a during SMC differentiation is a specific event which likely dependent on specific cellular context, miRNA binding sites and surrounding sequence, and/or the interactions between RNA binding proteins and 3'UTR of SirT1 gene

It has been suggested that miR-34a regulates cell cycle progress and apoptosis in cancer cells by repressing its target gene *SirT1* and forming a double positive feedback loop to regulate p53 activity[34, 35]. Surprisingly, we have provided clear and solid evidence to suggest that rather than translational repression, miR-34a regulates SirT1 in a translational activation manner. However, it is noteworthy to point out that

the up-regulation of SirT1 expression by miR-34a during SMC differentiation from ES cells is representing a specific event between miR-34a and SirT1 in our SMC differentiation model, which is likely dependent on specific cellular context, miRNA binding sites and surrounding sequence, and/or other RNA binding proteins. Such notion has been supported by several lines of evidence: Firstly, *SirT1* gene expression was significantly activated during SMC differentiation from stem cells (**Figure 3B**). Secondly, *SirT1* gene and protein expression levels were positively regulated by miR-34a expression levels as demonstrated in miR-34a over-expression and inhibition experiments (**Figure 3C and D**). Thirdly, over-expression of miR-34a significantly up-regulates *SirT1* 3'UTR activity, but such up-regulation was completely abolished when the miR-34a binging sites were mutated (**Figure 3E**). Fourthly, the luciferase activity of other 3'UTR reporters, which containing the binding site(s) of unrelated miRNAs, but not of miR-34a, were not regulated by miR-34a (**Figure S6A**). Fifthly, *SirT1* gene expression levels in the Matrigel implants with miR-34a over-expressing ES cells were much higher than that of the Matrigel plugs implanted with control ES cells (**Figure 2C and 2D**), suggesting *SirT1* gene expression level is also positively associated with miR-34a expression level during *in vivo* SMC differentiation from stem cells. Finally but importantly, we observed a negative regulatory relationship between a non-miR-34a reporter (pmiR-luc-ZEB1) and its corresponding miRNA (miR-200c) (**Figure S6B and S6C**). The unexpected but novel finding by which miR-34a positively regulates SirT1 could be explained by the following facts and/or observations: 1) Differentiating ES cells undergo cell cycle arrest at G0/1 phase and miR-34a promotes cell cycle arrest at G0/1 phase (**Figure S7A and S7B**). As mentioned early, the miRNA-target gene regulatory machinery will switch from translation repression to activation when the cells have been arrested at G0/1 phase[10] and locked in quiescent cells[11]. Therefore, our findings that miR-34a positively regulates target gene SirT1 during SMC differentiation is consistent with their data. 2) Another suggested criteria by the study[10] for miRNAs up-regulate their target genes is the AU rich elements existing within 3'UTR. By using free online search tool (**http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi**), similar to TNF-α we have found that SirT1 3'UTR is an A/U rich sequence (67%), and identified nearly 11 AU rich elements (ARE) sites within 3'UTR of SirT1 (spanning through 1000bps). Most importantly, we have also found the miR-34a binding site 2 is located closely with 8 of 11 ARE sites within SirT1 3'UTR (**Figure S9**). 3) miR-34a negatively regulates

SirT1 gene expression in undifferentiated ES cells, while positively regulates SirT1 gene expression in the terminally differentiated SMCs (**Figure 4A and 4B**). 4) We observed that G0/G1 cell cycle arrest inducer aphidicolin could up-regulate SirT1 gene reporter activity, and a synergetic effect between aphidicolin and miR-34a over-expression on SirT1 gene regulation (**Figure S7F**). 5) Removal of surrounding RNA sequence and structure of SirT1 3'UTR almost abolished the up-regulative effects of miR-34a on SirT1 3'UTR reporter activity (**Figure 4C**). All these observations have provided a strong indication that miR-34a may positively regulate SirT1 gene expression through their binding sites within 3'UTR, which has been nicely proven by our finding that miR-34a binding site 2, rather than binding site 1, is required for miR-34a mediated SirT1 gene up-regulation (**Figure 3E**). Our data also clearly suggests that up-regulation of SirT1 by miR-34a during SMC differentiation is a specific event (G0/G1 cell cycle arrest) between miR-34a and SirT1 gene during SMC differentiation, and both miR-34a binging sites and surrounding RNA sequence/structures within 3'UTR of SirT1 gene are required for such an event. Evidently, data from other studies also suggests that miRNAs can up-regulate their target genes in other cell systems under normal cell culture condition. It have been reported that miR-155 directly and indirectly enhances its target gene *TNF-α* translation in macrophages[36], and miR-744 up-regulates *ccnB1* through the binding sites within gene promoter region in NIH/3T3 fibroblasts as well as TRAMP C1 cells[37]. Additionally, miRNAs have also been suggested to up-regulate their target genes in immature oocytes[38].

It has been well-documented in the literature that various RNA-binding proteins (RBPs) play a crucial role in modulating miRNA function. An elegant study conducted by Kundu and colleagues has nicely demonstrated that RNA-binding protein HuR attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA[39]. Moreover, our previous studies have suggested an important role for another two RBPs, hnRNPA1[3] and hnRNPA2B1[40], in SMC differentiation from stem cells. Importantly, our data shown in **figure 4C** suggested that miR-34a mediates SirT1 up-regulation during SMC differentiation in a sequence dependent manner. Together with our another observation that much less miR-34a and SirT1 3'UTR were loaded into Ago 2 RISC complex upon SMC differentiation (**Figure 4 E**), which prompt us to speculate that miR-34a positively regulates its

target gene SirT1 during SMC differentiation through a similar mechanism as reported by Kundu and colleagues. In other words, the up-regulation of SirT1 by miR-34a may well be a result of RBPs, such as HuR, hnRNPA1 and hnRNPA2B1, interacting with miR-34a binding sites and/or other elements within the 3'UTR of SirT1 to disengage it from Ago2-RISC complex during differentiation. Although it warrants for further studying, investigations into confirming such a possibility is outside the current remit of this study.

SirT1 regulates SMC differentiation gene expression through a transcriptional mechanism

One of novel mechanistic findings in the present study is that we provided clear evidence to support that SirT1 regulates SMC differentiation gene expression through a transcriptional mechanism. SirT1 is a member of the a NAD+-dependent class III group of histone deacetylases, and has been reported to be involved in a variety of biological systems and cellular functions, including obesity-associated metabolic diseases, cancer, aging, cellular senescence, cardiac aging and stress, prion-mediated neurodegeneration, inflammation, and placental cell survival[41]. Importantly, recent data also suggests that SirT1 is a critical mediator in regulation of various development genes during stem cell differentiation[42], and plays an important role in various cellular differentiations including endothelial progenitor cells[43], hematopoietic cells[44], and osteoblasts[45]. In the present study, we have provided clear evidence that SirT1 is an important SMC differentiation mediator by transcriptional regulation of SMC specific genes and transcriptional factors. Traditionally, SirT1 has mainly been linked to negative regulation of gene expression through deacetylation of histone and non-histone proteins[46], however, accumulating evidence also strongly suggests that SirT1 can act both positively and negatively to control gene expression by recruiting a different set of coactivators and corepressors such as peroxisome proliferator–activated receptor γ coactivator-1α[47] or via a transcriptional mechanism[48]. In response to stress, activated SirT1 transcriptionally regulates its downstream target genes such as transcriptional factors (forkhead box Os and hypoxia-inducible factors-2α) and cardiac α-myosin heavy chain, through which SirT1 exerts a cardioprection response[41]. In consistent with these findings, we have provided strong evidence in the current study which firmly demonstrated that SirT1 is a transcription activator in regulation of SMC-specific gene expression (*SMαA, SM22α, SRF, MEF2c* and *myocd*) during SMC differentiation (**Figure 6A and 7B**). Direct evidence for such

transcriptional regulation of SMC specific genes was obtained from CHIP assays (**Figure 6B and 7C**), in which our data revealed that SirT1 can directly bind to the promoter DNA of SMC-specific genes and transcription factors.

Another important finding of the present study is that we further demonstrate that SirT1 acts as a potential transcriptional regulator for SMC gene regulation through modulating epigenetic modifications. It has been reported that SirT1 can induce the production of H3K9me3 in cultured U2OS cells[49] through directly interacting with and deacetylating histone methyltransferase SUV39H1[50]. Our previous data also suggests that H3K9 methylation was enriched within SMC specific gene promoter regions in the differentiating stem cells[5]. Data in the current study supports a notion that instead of inducing H3K9me production, SirT1 represses H3K9 tri-methylation status within SMC-specific gene promoters, resulting in SMC gene activation. However, the functional involvements of SirT1 in the regulation of other epigenetic modifications remain to be fully elucidated.

Taken together, we have successful uncovered a novel role of miR-34a in SMC differentiation from stem cells *in vitro* and *in vivo,* and provided compelling evidence to support our unexpected finding that miR-34a positively regulates its target gene SirT1 during SMC differentiation. Moreover, our data also suggests that the miRNA-target gene regulatory machinery should be carefully considered under certain circumstance. Furthermore, we have revealed that SirT1 regulates SMC gene expression through a transcriptional mechanism as well as an epigenetic signal pathway. However, it is noteworthy to mention that although we have provided some direct and strong evidence to show that SirT1 regulates SMC-specific transcription factor gene transcriptional activity through direct binding to the promoter regions of these genes (*SRF*, *Myocd* and *MEF2c*) (**Figure 7**), the minimal essential binding elements of SirT1 or exactly SirT1 binding motifs within these gene promoter regions remains to be further identified in our future study. Nevertheless, our findings will significantly increase our understanding of the molecular mechanisms in SMC differentiation and benefit future application in regenerative medicine.

Materials and Methods

ES cell culture and smooth muscle cell (SMC) differentiation. Detailed protocols for mouse embryonic stem cells (mESCs) (ES-D3 cell line, CRL-1934; ATCC, Manassas, USA) culture and SMC differentiation were described in our previous studies[3-6, 19, 24, 40, 51, 52]. The Shef-1, 2, 3, 6 and 7 human embryonic stem (ES) cell lines were obtained from the United Kingdom Stem Cell Bank (UKSCB, Hertfordshire, UK) and maintained in our Laboratory as described in our previous study[20]. Briefly, undifferentiated ES cells were dissociated into single cells and seeded onto collagen I/IV (5μg/ml)-coated flasks or plates in differentiation medium [DM, MEM alpha medium (Gibco) supplemented with 10% FBS, 0.05mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 0 to 9 days prior to further treatment. The medium was refreshed every other day. In our preliminary studies, we observed that there were no significant differences in terms of SMC differentiation capacity among these cell lines, but the Shef-3 cell line was the best to grow and scale-up. Therefore, Shef-3 cell line was routinely maintained in our laboratory and used in the current study.

microRNA and plasmids transfection. Either miRNAs inhibitors or precursors and miRNA negative controls (30nM) were transfected into differentiating ES cells using siPORT^{TM} NeoFX^{TM} transfection agent (Ambion, Applied Biosystems) according to the manufacturer's instructions. Transfected cells were plated into flasks or plates coated with 5μg/ml of collagen and cultured for 48~72 hours in the SMC differentiation medium to allow SMC differentiation. All miRNAs inhibitors or precursors and respective negative controls were purchased from Ambion. Control (pcDNA3.1) and SirT1 overexpression (pcDNA3.1-SirT1[21], a kind gift from Dr. Hang Shi, Wake Forest University School of Medicine, North Carolina, USA) plasmids were transfected into differentiating ES cells using Fugene-6 (Roche) according to the manufacturer's instructions.

SirT 3'UTR clone and miR-34a binding sites mutation. Reporter vector harbouring sequences of the murine SirT1 was created using cDNA from differentiating ES cells. The 3'-flanking untranslation region (2306bp/3636bp) of murine SirT1 gene (NM_019812.2) was amplified by PCR with primers shown in **Table S3** and cloned into the Mlu I and Sac I sites of the pmiR-reporter-basic vector (Ambion, Applied Biosystems), designated as pmiR-Luc-SirT1-WT. miR-34a binding site 1, 2 mutation

alone or combination were introduced into pmiR-Luc-SirT1 by using QuikChange™ site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. These were designated as pmiR-Luc-SirT1-BS1^{mu}, pmiR-Luc-SirT1-BS2^{mu}, and pmiR-Luc-SirT1-BS1/2^{mu} mutants, respectively. An artificial miR-34a binding site was introduced into pmiR-Luc by using PCR based QuikChange™ site-directed mutagenesis, designated as pmiR-Luc-miR-34a binding site. All vectors were verified by DNA sequencing.

Statistical analysis. Data were expressed as mean±SEM and analyzed using a two-tailed student's *t*-test for two-group comparision or one-way ANOVA followed by Tukey's HSD multiple comparison post-hoc test for comparing different groups. A value of $P < 0.05$ was considered as statistically significant.

***A detailed description on the expanded materials and methods is provided in the Online Supporting Information*.**

Acknowledgments

We are grateful to the supports from British Heart Foundation (FS/09/044/28007, PG/11/40/28891 and PG/13/45/30326), National Natural Science Foundation of China (91339102, 81270001 and 81270180) and Zhejiang Provincial Natural Science Foundation (LR14H020001). This work forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit which is supported and funded by the National Institute of Health Research.

Conflict-of-interest disclosure:

None

References:

1. Xiao Q, Zeng L, Zhang Z, Margariti A, Ali ZA, Channon KM *et al.* Sca-1+ progenitors derived from embryonic stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. *Arterioscler Thromb Vasc Biol* 2006; **26**(10)**:** 2244-51.

2. Zeng L, Xiao Q, Margariti A, Zhang Z, Zampetaki A, Patel S *et al.* HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. *J Cell Biol* 2006; **174**(7)**:** 1059-69.

3. Huang Y, Lin L, Yu X, Wen G, Pu X, Zhao H *et al.* Functional involvements of heterogeneous nuclear ribonucleoprotein A1 in smooth muscle differentiation from stem cells in vitro and in vivo. *Stem Cells* 2013; **31**(5)**:** 906-17.

4. Xiao Q, Pepe AE, Wang G, Luo Z, Zhang L, Zeng L *et al.* Nrf3-Pla2g7 interaction plays an essential role in smooth muscle differentiation from stem cells. *Arterioscler Thromb Vasc Biol* 2012; **32**(3)**:** 730-44.

5. Xiao Q, Wang G, Yin X, Luo Z, Margariti A, Zeng L *et al.* Chromobox protein homolog 3 is essential for stem cell differentiation to smooth muscles in vitro and in embryonic arteriogenesis. *Arterioscler Thromb Vasc Biol* 2011; **31**(8)**:** 1842-52.

6. Pepe AE, Xiao Q, Zampetaki A, Zhang Z, Kobayashi A, Hu Y *et al.* Crucial role of nrf3 in smooth muscle cell differentiation from stem cells. *Circ Res* 2010; **106**(5)**:** 870-9.

7. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R *et al.* Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 2005; **122**(4)**:** 553-63.

8. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K *et al.* Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* 2006; **312**(5770)**:** 75-9.

9. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**(2)**:** 102-14.

10. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science* 2007; **318**(5858)**:** 1931-4.

11. Vasudevan S, Tong Y, Steitz JA. Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle* 2008; **7**(11)**:** 1545-9.

12. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007; **129**(7)**:** 1401-14.

13. Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci U S A* 2005; **102**(34)**:** 12135-40.

14. Wang Y, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* 2007; **39**(3)**:** 380-5.

15. Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem* 2005; **280**(10)**:** 9330-5.

16. Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. *Arterioscler Thromb Vasc Biol* 2010; **30**(6)**:** 1118-26.

17. Houbaviy HB, Murray MF, Sharp PA. Embryonic stem cell-specific MicroRNAs. *Dev Cell* 2003; **5**(2)**:** 351-8.

18. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y *et al.* A microRNA component of the p53 tumour suppressor network. *Nature* 2007; **447**(7148)**:** 1130-4.

19. Xiao Q, Luo Z, Pepe AE, Margariti A, Zeng L, Xu Q. Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H2O2. *Am J Physiol Cell Physiol* 2009; **296**(4)**:** C711-23.

20. Luo Z, Wen G, Wang G, Pu X, Ye S, Xu Q *et al.* MicroRNA-200C and -150 play an important role in endothelial cell differentiation and vasculogenesis by targeting transcription repressor ZEB1. *Stem Cells* 2013; **31**(9)**:** 1749-62.

21. Yang Z, Kahn BB, Shi H, Xue BZ. Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. *J Biol Chem* 2010; **285**(25)**:** 19051-9.

22. Shakibaei M, Shayan P, Busch F, Aldinger C, Buhrmann C, Lueders C *et al.* Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: potential role of Runx2 deacetylation. *PLoS One* 2012; **7**(4)**:** e35712.

23. Tseng PC, Hou SM, Chen RJ, Peng HW, Hsieh CF, Kuo ML *et al.* Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. *J Bone Miner Res* 2011; **26**(10)**:** 2552-63.

24. Margariti A, Xiao Q, Zampetaki A, Zhang Z, Li H, Martin D *et al.* Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells. *J Cell Sci* 2009; **122**(Pt 4)**:** 460-70.

25. Miano JM. Serum response factor: toggling between disparate programs of gene expression. *J Mol Cell Cardiol* 2003; **35**(6)**:** 577-93.

26. Rosenfeld JA, Wang Z, Schones DE, Zhao K, DeSalle R, Zhang MQ. Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics* 2009; **10:** 143.

27. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ* 2010; **17**(2)**:** 193-9.

28. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH *et al.* Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 2007; **26**(5)**:** 745-52.

29. Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N *et al.* Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007; **26**(5)**:** 731-43.

30. Choi YJ, Lin CP, Ho JJ, He X, Okada N, Bu P *et al.* miR-34 miRNAs provide a barrier for somatic cell reprogramming. *Nat Cell Biol* 2011; **13**(11)**:** 1353-60.

31. Aranha MM, Santos DM, Sola S, Steer CJ, Rodrigues CM. miR-34a regulates mouse neural stem cell differentiation. *PLoS One* 2011; **6**(8)**:** e21396.

32. Navarro F, Gutman D, Meire E, Caceres M, Rigoutsos I, Bentwich Z *et al.* miR-34a contributes to megakaryocytic differentiation of K562 cells independently of p53. *Blood* 2009; **114**(10)**:** 2181-92.

33. Bu P, Chen KY, Chen JH, Wang L, Walters J, Shin YJ *et al.* A microRNA miR-34a-regulated bimodal switch targets notch in colon cancer stem cells. *Cell Stem Cell* 2013; **12**(5)**:** 602-15.

34. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* 2008; **105**(36)**:** 13421-6.

35. Yamakuchi M, Lowenstein CJ. MiR-34, SIRT1 and p53: the feedback loop. *Cell Cycle* 2009; **8**(5)**:** 712-5.

36. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B *et al.* Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 2007; **179**(8)**:** 5082-9.

37. Huang V, Place RF, Portnoy V, Wang J, Qi Z, Jia Z *et al.* Upregulation of Cyclin B1 by miRNA and its implications in cancer. *Nucleic Acids Res* 2012; **40**(4)**:** 1695-707.

38. Mortensen RD, Serra M, Steitz JA, Vasudevan S. Posttranscriptional activation of gene expression in Xenopus laevis oocytes by microRNA-protein complexes (microRNPs). *Proc Natl Acad Sci U S A* 2011; **108**(20)**:** 8281-6.

39. Kundu P, Fabian MR, Sonenberg N, Bhattacharyya SN, Filipowicz W. HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. *Nucleic Acids Res* 2012; **40**(11)**:** 5088-100.

40. Wang G, Xiao Q, Luo Z, Ye S, Xu Q. Functional impact of heterogeneous nuclear ribonucleoprotein A2/B1 in smooth muscle differentiation from stem cells and embryonic arteriogenesis. *J Biol Chem* 2012; **287**(4)**:** 2896-906.

41. Yang Yang WD, Yue Li, Zhenxiao Jin, Juanjuan Yan, Shiqiang Yu and Dinghua Yi. Novel Role of Silent Information Regulator 1 in Myocardial Ischemia. *Circulation* 2013; **128:** 2232-2240.

42. Calvanese V, Lara E, Suarez-Alvarez B, Abu Dawud R, Vazquez-Chantada M, Martinez-Chantar ML *et al.* Sirtuin 1 regulation of developmental genes during differentiation of stem cells. *Proc Natl Acad Sci U S A* 2010; **107**(31)**:** 13736-41.

43. Cheng BB, Yan ZQ, Yao QP, Shen BR, Wang JY, Gao LZ *et al.* Association of SIRT1 expression with shear stress induced endothelial progenitor cell differentiation. *J Cell Biochem* 2012; **113**(12)**:** 3663-71.

44. Ou X, Chae HD, Wang RH, Shelley WC, Cooper S, Taylor T *et al.* SIRT1 deficiency compromises mouse embryonic stem cell hematopoietic differentiation, and embryonic and adult hematopoiesis in the mouse. *Blood* 2011; **117**(2)**:** 440-50.

45. Srivastava S, Bedi U, Roy P. Synergistic actions of insulin-sensitive and Sirt1-mediated pathways in the differentiation of mouse embryonic stem cells to osteoblast. *Mol Cell Endocrinol* 2012; **361**(1-2)**:** 153-64.

46. Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* 2001; **410**(6825)**:** 227-30.

47. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 2005; **434**(7029)**:** 113-8.

48. Chang HC, Guarente L. SIRT1 mediates central circadian control in the SCN by a mechanism that decays with aging. *Cell* 2013; **153**(7)**:** 1448-60.

49. Vaquero A, Scher M, Lee D, Erdjument-Bromage H, Tempst P, Reinberg D. Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol Cell* 2004; **16**(1)**:** 93-105.

50. Vaquero A, Scher M, Erdjument-Bromage H, Tempst P, Serrano L, Reinberg D. SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature* 2007; **450**(7168)**:** 440-4.

51. Zhang L, Jin M, Margariti A, Wang G, Luo Z, Zampetaki A *et al.* Sp1-dependent activation of HDAC7 is required for platelet-derived growth factor-BB-induced smooth muscle cell differentiation from stem cells. *J Biol Chem* 2010; **285**(49)**:** 38463-72.

52. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. *Am J Physiol Cell Physiol* 2007; **292**(1)**:** C342-52.

Figure Legend

Figure 1. Important role of miR-34a in SMC differentiation from ES cells.

(A) miR-34a was up-regulated during SMC differentiation from ES cells. Day 0 samples were undifferentiated ES cells and served as negative control. (B-C) miR-34a overexpression significantly increases SMC marker expressions. ES cells were transfected with miR-34a precursor or miR precursor negative control, and cultured in SMC differentiation medium for 48~72 hours. miR-145 precursor in (C) was included as positive control. (D-E) miR-34a inhibition reduces SMC marker expressions. Day 3 differentiating ES cells were transfected with miR-34a inhibitor or miR inhibitor negative control, and cultured in SMC differentiation medium for 48 hours. miR-145 inhibitor in (D-E) was included as positive control. The data presented here are representative or mean ± S.E.M. of three to six independent experiments. *P<0.05.

Figure 2. miR-34a promotes SMC differentiation *in vivo*.

Matrigel plugs implanted with ES cells without (pLL3.7-GFP) or with (pLL3.7-GFP-miR-34a) miR-34a over-expression were harvested, sectioned and subjected to immunofluorescence staining using antibodies against GFP and SM-MHC, or SirT1 alone. Representative images (A) and quantitative data (B) of the percentage of SM-MHC-positive cells were presented here, respectively. Note: cells with green fluorescence signal indicate GFP-positive cells (implanted cells) within Matrigel plugs. The percentage of GFP-labelled SM-MHC-positive cells per field were examined by two well-trained independent investigators blinded to the treatments, from four random high power fields (200x) in each section, three sections from each implant and four implants for each group, *p<0.05. (C-D) Gene expression levels within Matrigel implants. Total RNA and protein samples were extracted from partial Matrigel implants and subjected to RT-qPCR (C) and western blot (D) analysis, respectively. The data presented here are representative or mean ± S.E.M. of four Matrigel implants. *P<0.05.

Figure 3. Target gene, SirT1, is positively regulated by miR-34a through the binding sites within 3'UTR.

(A) The potential binding sites of miR-34a within SirT1 3'UTR as predicted by *Targetscan* are depicted in this illustration. (B) SirT1 was up-regulated during SMC differentiation from ES cells. (C and D) Modulations of miR-34a expression levels positive regulate SirT1. (E) miR-34a binding site 2 is required for miR-34a mediated SirT1 gene activation. miR-34a precursor or negative control and wild type SirT1

3'UTR reporter (pmiR-Luc-SirT1-WT) or three mutants [bindings site 1 ($BS1^{mu}$), 2 ($BS2^{mu}$), and combinational mutations ($BS1/2^{mu}$)] were co-transfected into day 2~3 differentiating ES cells and luciferase activity assay were measured at 48 hours post-transfection. The data presented here are representative or mean±S.E.M. of three to four independent experiments. *P<0.05 (treatment versus day 0 or control), #P<0.05 in (E) (binding site mutants versus wild type).

Figure 4. miR-34a differently regulates SirT1 gene expression in ES cells and terminally differentiated SMCs

(A) miR-34a negatively regulates SirT1 expression in naive stem cell stage. Undifferentiated ES cells were co-transfected with control miRNAs, miR-34a precursor or miR-34a inhibitor and pmiR-Luc-SirT1, respectively. Luciferase activity assay were measured at 48 hours post-transfection. (B) miR-34a positively regulates SirT1 expression in terminally differentiated stage. Day 8 differentiated SMCs were co-transfected with control miRNAs, miR-34a precursor or miR-34a inhibitor and pmiR-Luc-SirT1, respectively. Luciferase activity assay were measured at 48 hours post-transfection. (C) Surrounding structure(s) or sequences of SirT1 3'UTR is required for miR-34a mediated SirT1 gene up-regulation. (D) Ago2 protein levels and IP efficiency. The expression levels of Ago2 (input) and RNA-IP efficiencies (Ago2-IP and IgG-IP) in undifferentiated ES cells (ESCs) and differentiated SMCs (dSMCs) were examined using Ago2 antibody. (E) Less miR-34a and SirT1 mRNA were incorporated into Ago2-RISC complex upon differentiation. Undifferentiated ES cells and differentiated SMCs were harvested and subjected to RNA-IP assay. RNA-IP assays were performed using antibodies against Ago2, or normal rabbit IgG, respectively, as described in online supplemental data. RT-qPCR amplification of the SirT1 coding region was included as additional control for specific SirT1 3'UTR enrichment. Dash line in (E) indicates the enrichment level for IgG control which was set as 1.0. The data presented here are representative or mean±S.E.M. of three to four independent experiments. *P<0.05 (vs. control).

Figure 5. SirT1 activation is required for miR-34a mediated SMC gene expressions.

(A-B) Over-expression of SirT1 up-regulated SMC gene expressions. Day 2~3 differentiating ES cells were transfected with control (pcDNA3.1) or SirT1 over-expression plasmid (pcDNA3.1-SirT1), and cultured in SMC differentiation medium

for another 48 or 72 hours. The data presented here are representative or mean± S.E.M. of three independent experiments, *P<0.05. (C) SirT1 inhibition abolished SMC gene expression increased by miR-34a over-expression. Control (pLL3.7-GFP) and miR-34a over-expressing (pLL3.7-GFP-miR-34a) ES cells were induced to differentiate into SMCs for 4 days and SirT1 specific inhibitor III (500nM, EMD Millipore, 566322) or control vehicle were added into the culture medium for 6 or 12 hours before harvest. (D) SirT1 knockdown abrogated the effects of miR-34a over-expression on SMC gene expression. Day 2~3 differentiating ES cells were co-transfected with miR-34a precursor, SirT1 siRNAs or respective control (miRNA precursor negative control and siRNA control) as indicated, and cultured in SMC differentiation medium for another 48 hours. (E) SirT-1 agonist resveratrol promotes SMC gene expression. The data presented here are mean±S.E.M. of three or four independent experiments. *P<0.05 (versus related controls); #P<0.05 (4^{th} bars versus 3^{rd} bars).

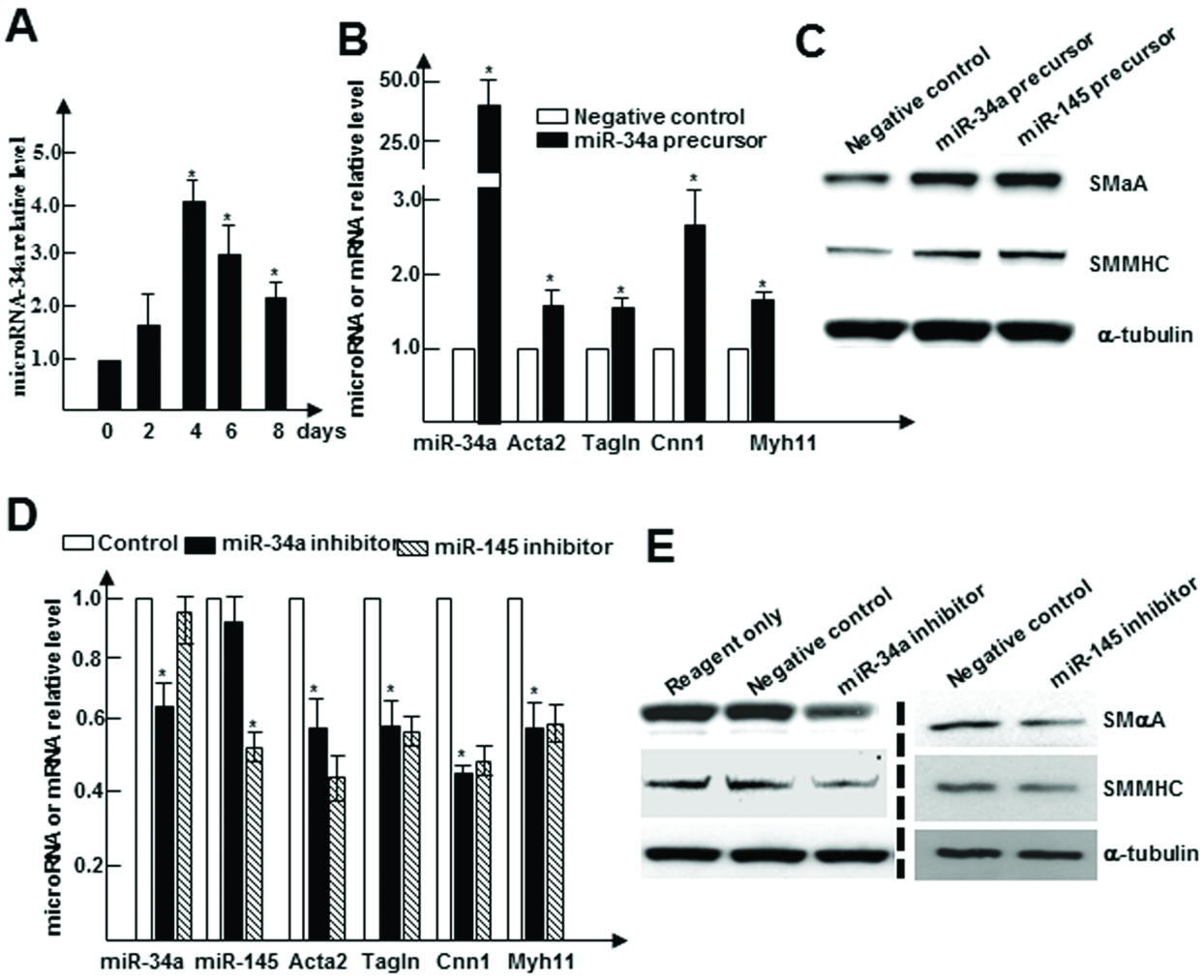
Figure 6. SRF binding site is required for SirT1 mediated SMC gene expression.

(A) SRF binding site mutation abolished SMC differentiation gene promoter activity induced by SirT1 overexpression. (B) SirT1 binds directly to the promoter regions of SMC differentiation genes. (C) Up-regulated SirT1 increases SRF binding to the promoter regions of SMC differentiation genes. ChIP assays were performed using antibodies against SirT1, SRF, or normal rabbit IgG, respectively, as described in online supplemental data. PCR amplifications of the non-CArG regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of four independent experiments. *P<0.05 (vs. control).

Figure 7. SMC transcription factor gene expressions were positively and transcriptionally regulated by SirT1.

(A) mRNA levels of *SRF*, *MEF2c* and *myocd* were significantly up-regulated by SirT1 over-expression. Total RNAs were harvested as described in Figure 5A. (B) SirT1 regulated the promoter activities of SMC transcription factor genes. Day 2~3 differentiating ES cells were transfected with luciferase reporter plasmids pGL3-SRF-Luc, pGL3-MEF2c-Luc or pGL3-Myocd-Luc (0.15μg/2.5×10^4 cells) together with pcDNA3.1-SirT1 or pcDNA3.1 (0.2μg/2.5×10^4 cells). pShuttle-LacZ (0.2μg/2.5×10^4cells) was included as control. Luciferase and β-galactosidase activity assays were detected 48 hours after transfection. The data presented here are

mean±S.E.M. of three to six independent experiments. *P<0.05 (vs. control). (C) SirT1 binds directly to the promoter regions of SRF, MEF2c and myocardin genes. ChIP assays were performed using antibodies against SirT1 or normal rabbit IgG, respectively, as described in online supplemental data. PCR amplifications of the adjacent regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of four independent experiments. *P<0.05 (vs. control).


A
microRNA-34a relative level
5.0
4.0
3.0
2.0
1.0
0 2 4 6 8 days
B
microRNA or mRNA relative level
50.0
25.0
3.0
2.0
1.0
Negative control
miR-34a precursor
miR-34a Acta2 Tagln Cnn1 Myh11
C
Negative control
miR-34a precursor
miR-145 precursor
SMaA
SMMHC
α-tubulin
D
Control
miR-34a inhibitor
miR-145 inhibitor
microRNA or mRNA relative level
1.0
0.8
0.6
0.4
0.2
miR-34a miR-145 Acta2 Tagln Cnn1 Myh11
E
Reagent only
Negative control
miR-34a inhibitor
Negative control
miR-145 inhibitor
SMαA
SMMHC
α-tubulin

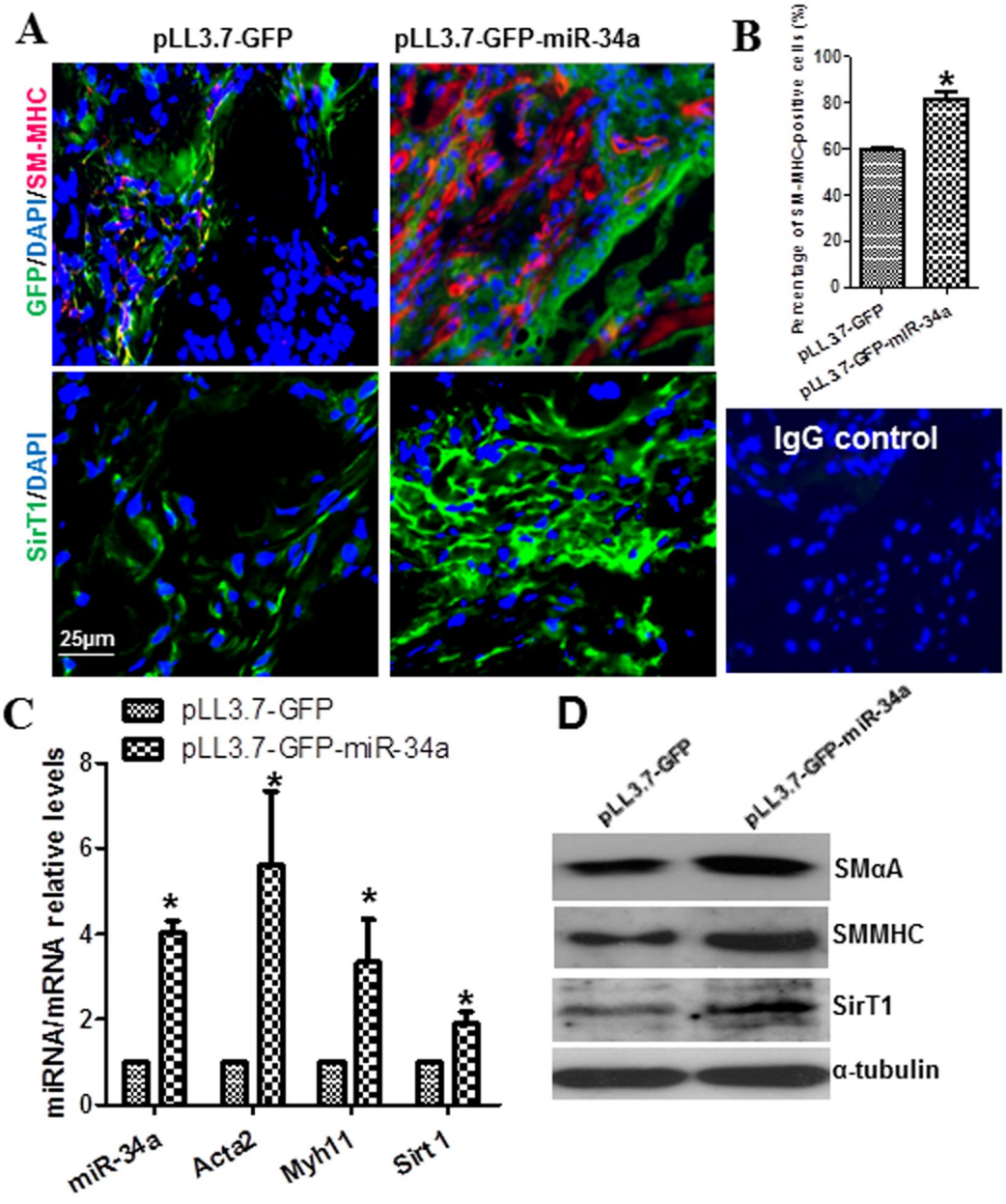
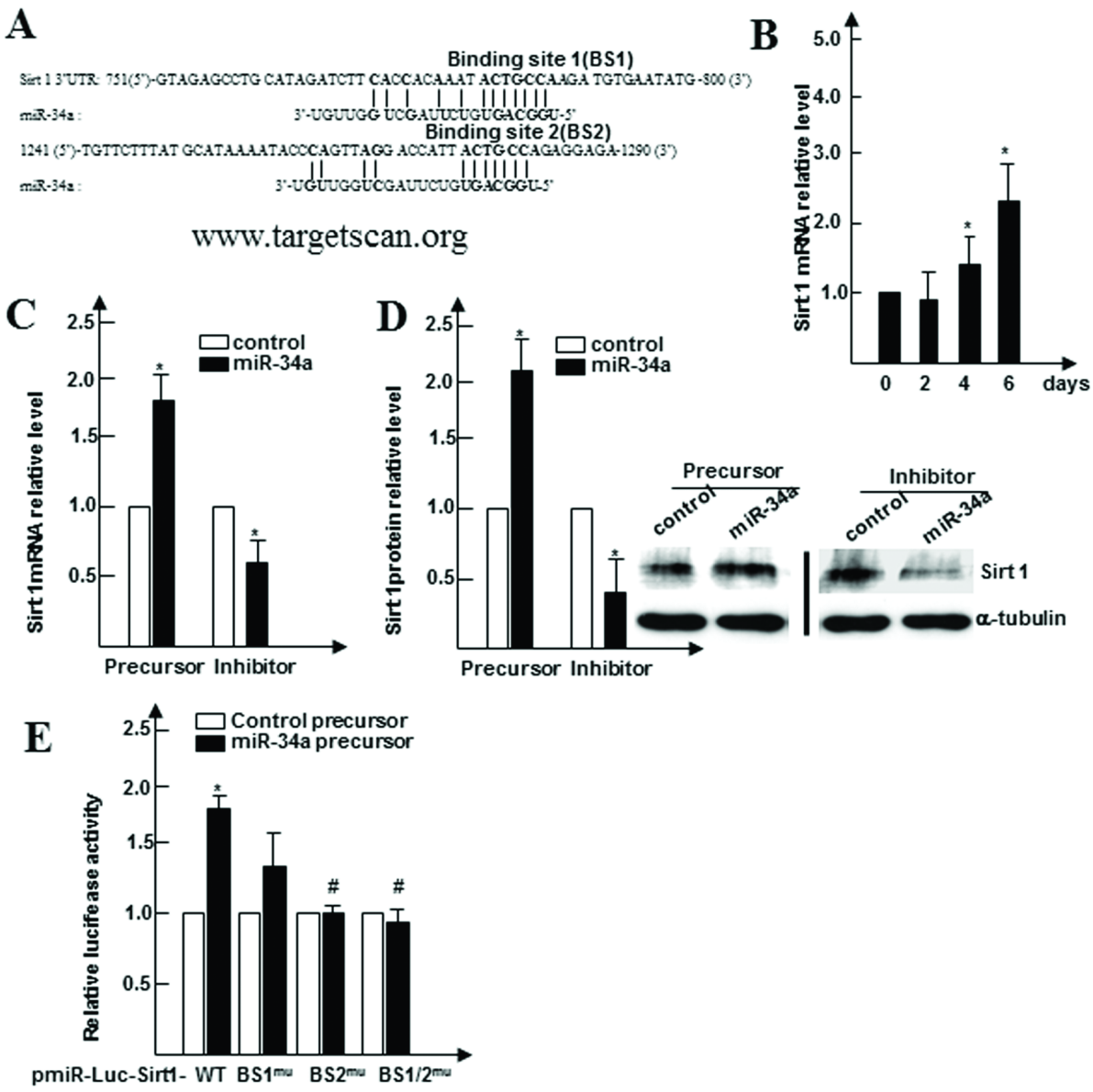

A
pLL3.7-GFP
pLL3.7-GFP-miR-34a
GFP/DAPI/SM-MHC
SirT1/DAPI
25μm
B
Percentage of SM-MHC-positive cells (%)
100
80
60
40
20
0
*
pLL3.7-GFP
pLL3.7-GFP-miR-34a
IgG control
C
pLL3.7-GFP
pLL3.7-GFP-miR-34a
miRNA/mRNA relative levels
8
6
4
2
0
*
miR-34a
Acta2
Myh11
Sirt 1
D
pLL3.7-GFP
pLL3.7-GFP-miR-34a
SMαA
SMMHC
SirT1
α-tubulin

Figure 3

Yu, et al



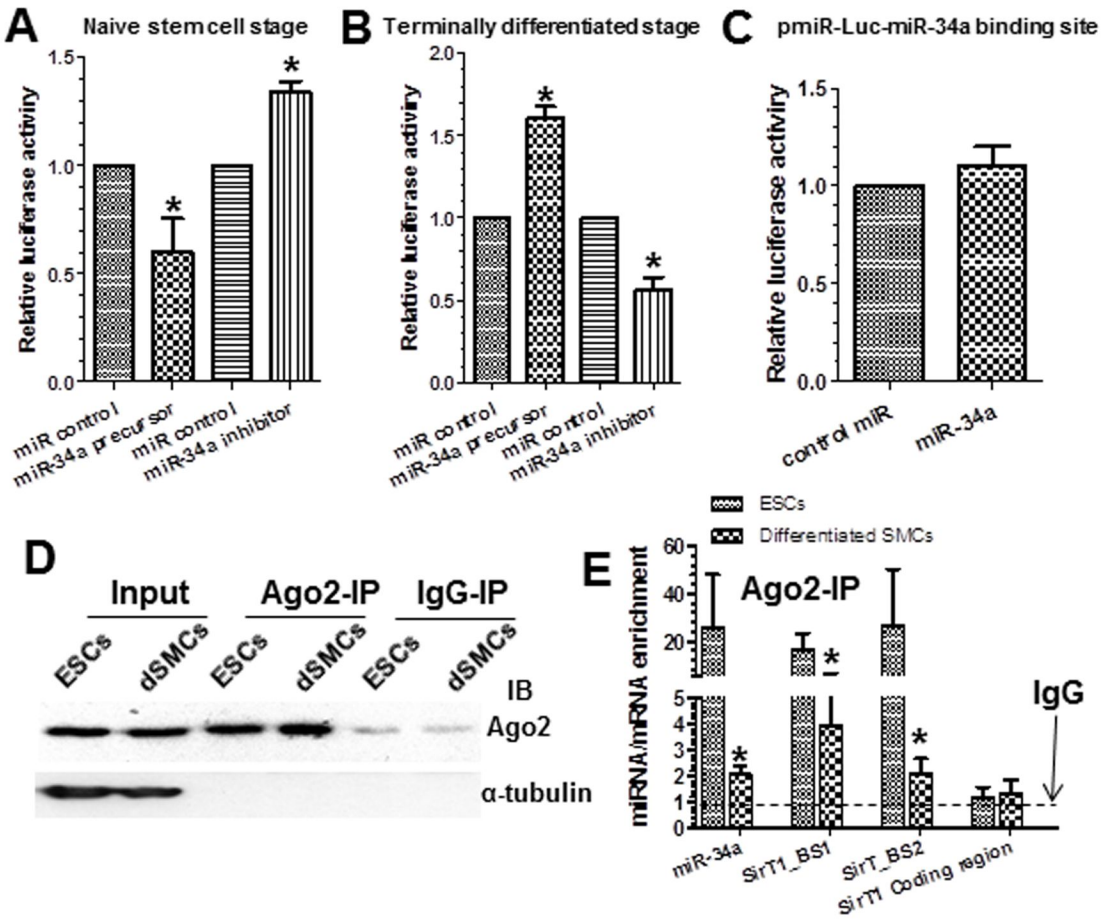
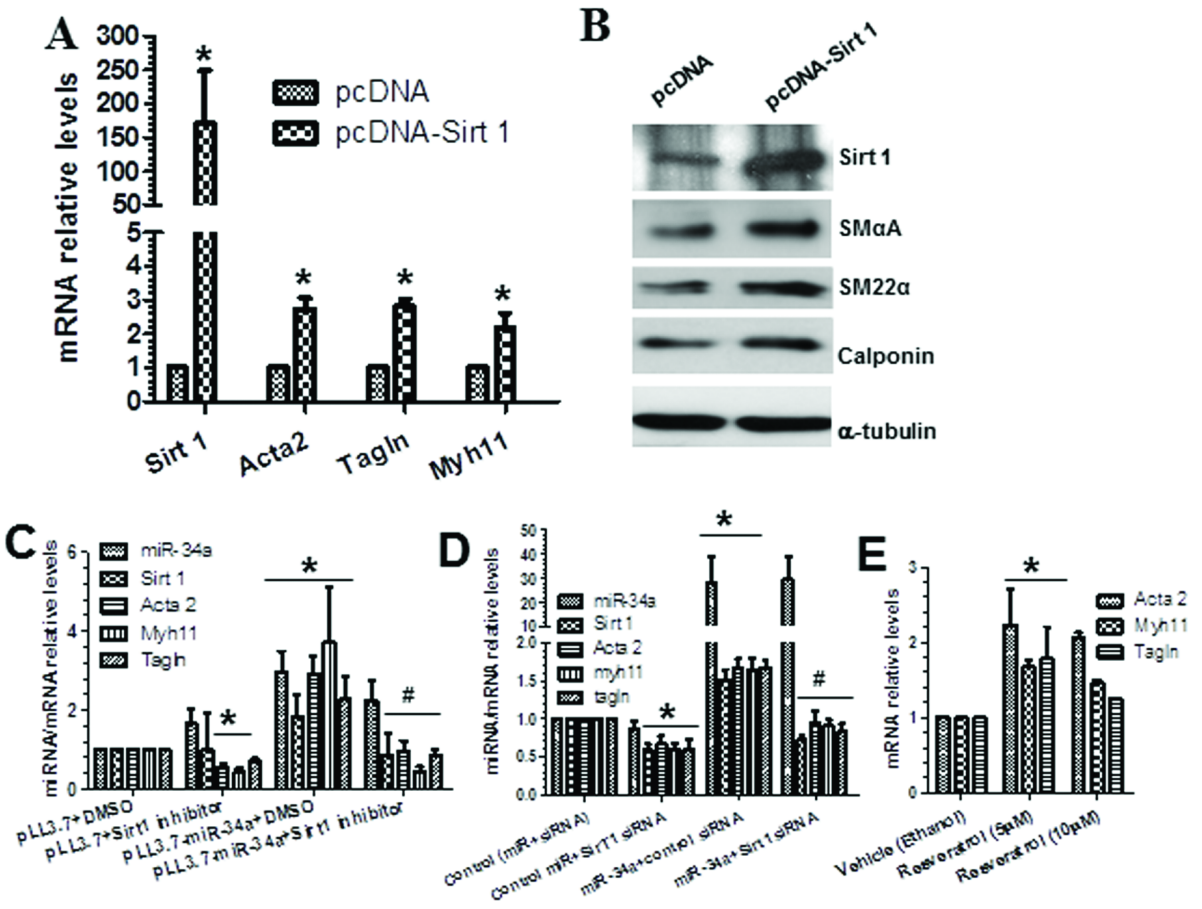

A
Naive stem cell stage
Relative luciferase activity
1.5
1.0
0.5
0.0
*
*
miR control
miR-34a precursor
miR control
miR-34a inhibitor
B
Terminally differentiated stage
Relative luciferase activity
2.0
1.5
1.0
0.5
0.0
*
*
miR control
miR-34a precursor
miR control
miR-34a inhibitor
C
pmiR-Luc-miR-34a binding site
Relative luciferase activity
1.5
1.0
0.5
0.0
control miR
miR-34a
D
Input
Ago2-IP
IgG-IP
ESCs
dSMCs
ESCs
dSMCs
ESCs
dSMCs
IB
Ago2
α-tubulin
E
ESCs
Differentiated SMCs
Ago2-IP
miRNA/mRNA enrichment
60
40
20
5
4
3
2
1
0
*
*
*
IgG
miR-34a
SirT1_BS1
SirT_BS2
SirT1 Coding region

Figure 5 ***Yu, et al***



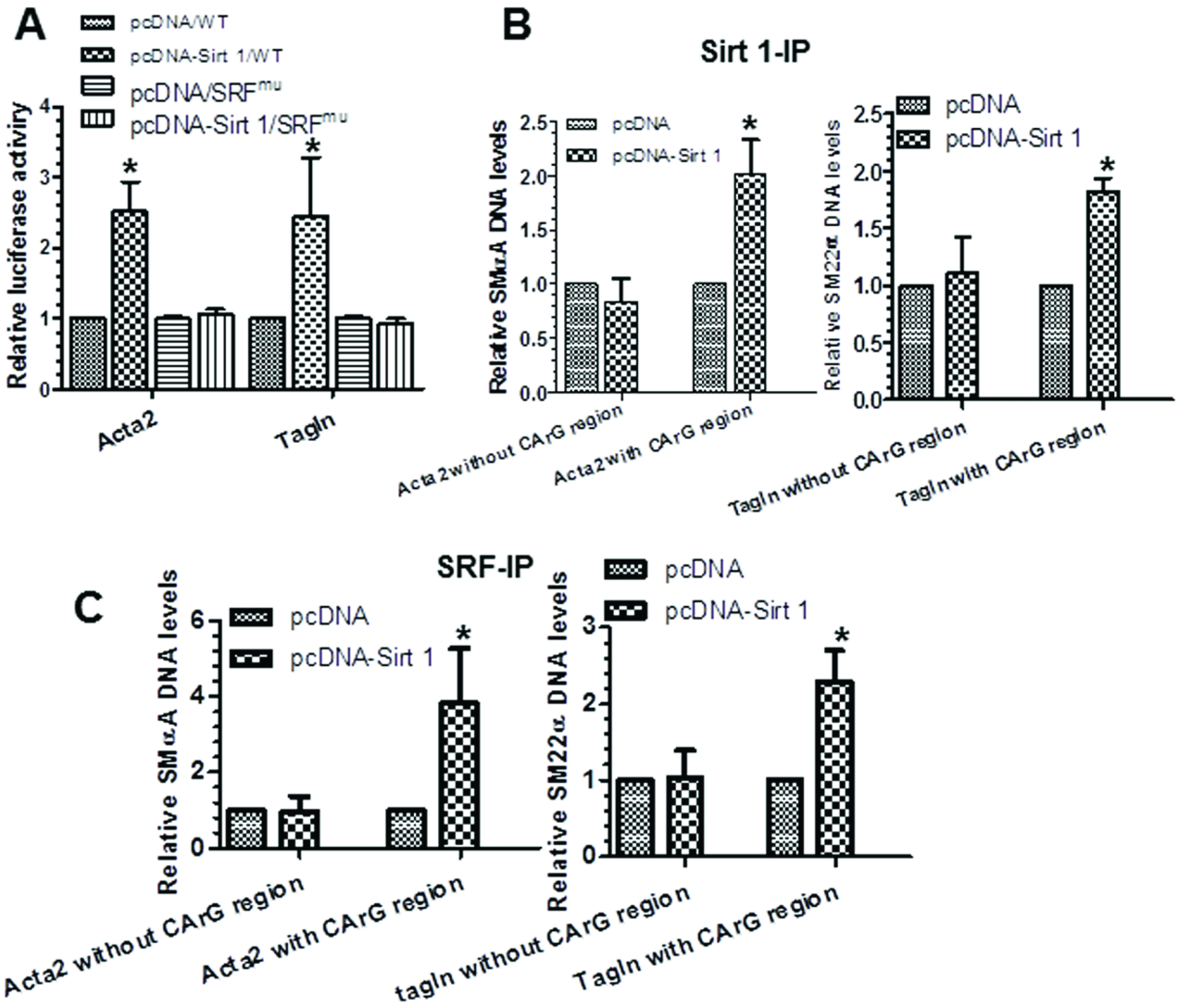

A
pcDNA/WT
pcDNA-Sirt 1/WT
pcDNA/SRF^mu
pcDNA-Sirt 1/SRF^mu
Relative luciferase activiry
Acta2
Tagln
B
Sirt 1-IP
pcDNA
pcDNA-Sirt 1
Relative SMαA DNA levels
Relative SM22α DNA levels
Acta2without CArG region
Acta2with CArG region
Tagln without CArG region
Tagln with CArG region
C
SRF-IP
pcDNA
pcDNA-Sirt 1
Relative SMαA DNA levels
Relative SM22α DNA levels
Acta2 without CArG region
Acta2 with CArG region
tagln without CArG region
Tagln with CArG region

Figure 7 ***Yu, et al***